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BINDING ASSAYS FOR THE QUANTITATIVE DETECTION OF P. BREVIS POLYETHER NEUROTOXINS IN BIOLOGICAL SAMPLES AND ANTIBODIES AS THERAPEUTIC AIDS FOR POLYETHER MARINE INTOXICATION

ANNUAL REPORT

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The polyether lipid-soluble toxins isolated from the marine dinoflagellate Ptychodiscus brevis can be detected using a number of different types of immunosorbant assay. A comparison of six different formats were undertaken, evaluating each of sensitivity and for background color development. Further purification of specific anti-brevetoxin antibody, followed by utilization in four different types of immunosorbant assay, indicated that a sandwich-type assay employing toxin adsorbed to plates, specific antibrevetoxin antibody, and antispecies-antibody covalently linked to peroxidase enzyme as primary, secondary, and tertiary adsorbant could yield sensitivities in the 40 pgram to 40 ngram concentration ranges. The stabilities of each of the reagents used in the latter "sandwich" assay were evaluated and checkerboard protocols were undertaken to optimize the assay. There is no cross-reactivity from the okadaic acid type polyether toxins, but substantial cross-reactivity with ciguatoxin. Final results will be summarized in the final report.

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Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) haved adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS Publication No. (NIH) 86-23, revised 1985).

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I. Statement of the Problem

This contract is concerned with the development of diagnostic methods and therapy for exposure to polyether toxins produced by marine dinoflagellates. We have proposed two separate binding assays which have potential value in quantitative detection of the toxins in biological samples. Our specific aims are to:

[1] develop and refine *in vitro* radiometric binding assays to detect polyether marine neurotoxins in biological samples using tritiated brevetoxin PbTx-3 (formerly T17) as radiometric probe and employing antibodies prepared in goat against PbTx-3 produced by laboratory cultures of *Ptychodiscus brevis* or synaptosomes prepared from rat brain;

[2] determine the sensitivity and specificity of the binding assays using brevetoxin standards mixed with biological samples of clinically-obtainable types, i.e. serum, mucousal secretions, urine and or feces;

[3] using goat antibodies or solubilized brevetoxin binding component from rat brain, develop enzyme-linked assays to further simplify the procedure for routine use;

[4] examine potential cross-reactivity of the binding assays with respect to other polyether toxins, and hence their usefullness in the detection of other lipid-soluble marine polyether toxins;

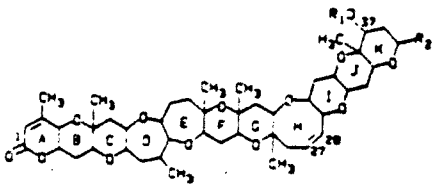
[5] examine the feasibility of using available antibodies as therapeutic agents, first using competitive *in vitro* molecular pharmacological binding assays, and later by examining the reversal of toxic effects in animals by immunoassay;

[6] provide reagents adequate for 10,000 assays, including radioactive toxin probe, and data on tests and evaluations. Detailed protocols will accompany reagents.

II . Background

A. Toxins

We routinely isolate six brevetoxins from laboratory cultures of P. brevis, all based on the two polyether backbones (1). In logarithmic cells, the two predominant toxins are PbTx-1 and PbTx-2 (see Figure 1). In stationary cells, approximately the same relative amounts of PbTx-1 and PbTx-2 are present on a per cell basis, but now in addition PbTx-3, PbTx-5, PbTx-6 (based on the backbone present in PbTx-2), and PbTx-7 (based on the backbone present in PbTx-1) appear. Two additional synthetic toxins, PbTx-9 and PbTx-10, are available by chemical reduction (2).



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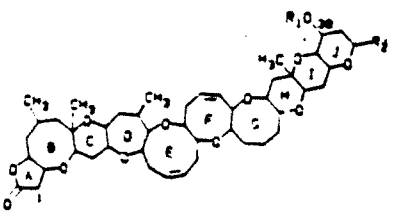

Type-2

Toxin	Type	R_1	R_2
PbTx-1	2	H	
PbTx-2	1	H	
PbTx-3	1	H	
PbTx-5	1		
PbTx-6	1	H	 27,28 epoxide
PbTx-7	2	H	
PbTx-8	1	H	
PbTx-9	1	H	
PbTx-10	2	H	

Figure 1. Structures of the Brevetoxins.

B. Molecular Pharmacology

High Affinity Binding Site. We have previously shown that PbTx-3 binds to site 5 associated with voltage-sensitive sodium channels, and have determined a KD of 2.9 nM and a Bmax of approximately 7 picomoles/mg synaptosomal protein for this site. We also demonstrated that tritiated PbTx-3 could be displaced in a specific manner from its binding site by either natural or synthetic brevetoxins. Our initial observation was that displacement efficiency was linked in a positive fashion with potency in animals. Specific displacement curves correlated well with the potency of each individual purified toxin. Differential lipid solubility of each of the natural brevetoxins made it imperative to include Emulphor EL-620 in all experimental tubes.

Species Similarity of Binding. Synaptosomes from rats, turtles, or fish were prepared to examine the binding characteristics of each with respect to brevetoxins. Table I outlines the results of the comparison, and illustrates that any of the three systems examined bind brevetoxins in a reproducible manner with approximately equal efficacy.

Table I. Comparison of Dissociation Constant (Kd) and Binding Maximum (Bmax) in Fish, Turtles, and Rats*

Species	Kd (nM)	Bmax (pMol/mg Protein)	Temp. Optimum (oC)	Specific Binding at Kd
Fish	6.1	1.40	23	80%
Turtle	1.5	2.25	4	80%
Rat	2.6	6.80	4	90%

*mean values for Kd and Bmax, n=9,4,6 for fish, turtles, and rats respectively.

In addition to developing displacement curves for the six toxins (n=2), we had sufficient toxin material for PbTx-1,-2,-3, and -7 to calculate Kis for each of the species. These are summarized for several species in Table II.

Table II. Inhibition Constants for Derivative Brevetoxins Derived from the Cheng-Prusoff* Equation

Toxin	Ki (nM) Turtle	Fish	Rat
PbTx-1	0.39	10.10	0.72
PbTx-2	1.34	23.57	3.51
PbTx-3	1.96	37.04	2.47
PbTx-5	----	----	2.68
PbTx-6	----	----	6.60
PbTx-7	----	----	0.85

*see Reference (3)

Specific binding of four tritiated brevetoxins in rat brain synaptosomes. A preliminary comparison of specific binding of tritiated PbTx-3, PbTx-7, PbTx-9, and PbTx-10 indicates an equivalent Bmax and a progression of Kd values which parallel the relative potencies of the labeled brevetoxins. This is a further indication to us that binding affinity is the conservative requirement in the potency of the brevetoxins (Table III), and further, that we may be able to utilize the toxins which are of higher specific activity for more detailed receptor characterization (4).

Table III. Comparison of Kd and Bmax for Four Different Tritiated Brevetoxin Probes in Rat Brain Synaptosomes

Toxin	Kd (nM)	Bmax (pmoles/mg protein)
PbTx-3	2.13	6.99
PbTx-9	8.76	6.75
PbTx-7	1.91	6.38
PbTx-10	1.56	6.46

Our evidence indicates that, at a Kd concentration of tritiated PbTx-3, the t1/2 for on- and off-rates approximate 1-2 minutes. A closer approximation cannot be derived utilizing present protocols. There is no membrane potential dependence of brevetoxin binding to the high affinity, low capacity binding site known as Site 5. Kd= 2.6 (intact), 2.9 (lysed), 3.3 (depolarized) and Bmax= 6.01 (intact), 5.83 (lysed) and 5.75 pmoles/mg protein (depolarized) (5).

Regardless of the organism used for synaptosomal preparations, it is apparent to us that the topographic characteristics of the brevetoxin binding site on the VSSC are comparable. Using brevetoxins PbTx-1-3, and PbTx-5-7, Ki data for specific displacement of tritiated PbTx-3 shows comparable data in each case (Table II in Introduction). The more hydrophobic type-2 brevetoxins are most efficacious in their ability to compete for site 5 binding (6).

Classes of brevetoxin binding sites. Two separate brevetoxin binding sites have been discovered in rat brain synaptosomes. The brevetoxins bind with an affinity constant which is consistently in the 1-5 nM concentration range, in good agreement with affinity data for other potent marine toxins like saxitoxin (7). In addition, the binding maximum in synaptosomes is also in good agreement with data for Site I toxins, which are known to bind to channels with a 1:1 stoiciometry. However, the allosteric modulation of sodium channel binding by other natural toxins by brevetoxins occurs at brevetoxin concentrations much higher, ca. 20-100 nM (8). This data is inconsistent with high affinity, low capacity binding.

Converse to this allosteric modulation which occurs at higher brevetoxin concentrations, is the finding that membrane depolarization, 22Na influx and competitive displacement of tritiated

brevetoxin binding by unlabeled competitors, is dose dependent in the same concentration ranges observed for the high affinity binding site (5). Thus, the allosteric modulation at other sodium channel binding sites appears to arise from brevetoxin interaction with a lower affinity, high capacity binding site.

Using classical Rosenthal analysis, we have been able to distinguish two separate specific brevetoxin sites (Table IV).

Table IV. The Two Brevetoxin Binding Sites

Site	Kd	Bmax	Allosteric Modulator
5	2.6-3.3	5.7-6.8	No
_*	79.1-300.	63.7-180	Yes

*not numbered until further work can be accomplished.

The two site hypothesis is supported by brevetoxin inhibition constant data and double reciprocal competition plots, which indicate a deviation from competitive type patterns to non-competitive type patterns at higher competitor brevetoxin concentrations. The non-competitive displacement appears to be specific in nature, and is not likely due to changes in membrane fluidity. Some evidence, see Results below, suggests that the low affinity binding site may be α- and/or β-Spectrin. Allosteric modulation of sodium channel function by Spectrin-type molecules has not been described.

C. Immunology

At a time when only the structures of PbTx-2 and PbTx-3 were known, we began developing immunoassays for the detection of brevetoxins in marine food sources (9). Utilizing bovine serum albumin-linked brevetoxin PbTx-3 as complete antigen, we succeeded in producing antiserum in a goat. We chose goats for the large quantities of immune serum which we could obtain, provided we could raise an antibody population. Subsequent characterization of the immune serum obtained indicated that both PbTx-2 and PbTx-3 were detected in approximately equivalent manners. Although oxidized PbTx-2 was not potent in either fish or mouse bioassay (5), it did displace PbTx-3 in competitive radioimmunoassay, an indication that potency was not reflected in RIA.

With the description of new brevetoxins based on the PbTx-2, PbTx-3 -type structural backbone (1), it was of interest to examine the competitive abilities of those new toxins. Based on the types of structural derivatives in tthis toxin series, we felt that new information regarding the epitopic sites on the brevetoxin backbone might be uncovered. In the same vein, the new structural backbone present in PbTx-1 and PbTx-7 might give us further insight into epitopic sites (the terminal 3 to 4 rings are identical) on brevetoxins.

Radioimmunoassay displacement curves indicated that the antibody recognizes and binds the toxins which possess the type of structure depicted on the left(type-1) of Figure 1 with much higher affinity than

it does the toxins whose backbone is illustrated in Figure 1 on the right (type-2). This is not surprizing because the antibody was produced by immunization with BSA-linked PbTx-3, a type-1 toxin (18). Statistical analysis of ED50 values reveal that there are no statistical differences between the efficiencies with which PbTx-2, PbTx-3, and PbTx-5 displace tritiated PbTx-3 from the antibody-hapten complex (t-test, $p<0.1$). Analysis of 50% displacement values for PbTx-1 and PbTx-7 (both type-2) revealed no statistically-significant difference ($p<0.001$). With the exception of PbTx-6, a significant difference was consistently found, however, between the curves for the two toxin backbones. Type-1 toxins are approximately 10-fold more efficient than are type-2 toxins at displacing tritiated PbTx-3 from the binding site. The exceptional case, PbTx-6, is a 27,28 epoxide of a type-1 toxin. An epitope on the toxin molecule may involve the configuration around the 27,28 carbon unsaturation (summarized in Table V) (6).

TABLE V. CORRELATION OF POTENCY WITH RADIOIMMUNOASSAY AND SYNAPTOSOME ASSAYS

Toxin	Synaptosome ED50 (nM)	Ki	LD50 (nM)	Radioimmunoassay ED50(nM)
PbTx-1	3.5	7.1	4.4	93.0
PbTx-7	4.1	8.9	4.9	92.0
PbTx-2	17.0	16.1	21.8	22.0
PbTx-3	12.0	37.0	10.9	20.0
PbTx-5	13.0	----	42.5	10.1
PbTx-6	32.0	----	35.0	112.0

ED50 are defined as the toxin conc at which 50% displacement of tritiated PbTx-3 from sodium channels or antibody occurs. LD50 are determined by incubation of Gambusia affinis with toxin in 20 mL seawater for 60 minutes. Ki are determined as described in the text.

In addition, we began to explore methods for converting the RIA to an enzyme linked form. We sought to use an enzyme system which was stable, produced a color reaction which would be visible to the naked eye (even though our evaluation would take place in a microtitre plate reader), would lend itself to coupling enzyme to either toxin or antibody, and would possess an enzymatic activity that was absent in mammalian systems (to reduce background color reactions).

The basic assay under development followed a noncompetitive enzyme immunoassay sandwich technique (figure 2). Heterogeneous system assays (7,8) such as these may be performed as either competitive or noncompetitive types, and may be either enzyme-antibody labeled or enzyme-hapten (antigen) labeled. Thus, the greatest flexibility is gained employing such techniques, and many different variations may be developed to meet defined criteria.

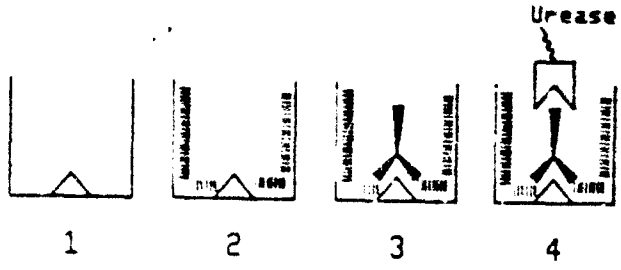

Figure 2. Non-Competitive Brevetoxin-Antibody-Protein A Urease ELISA.

In order for the proposed assay to work, toxin PbTx-2 (illustrated as triangles) had to be successfully bound to the microtitre plate wells. Unlike standard enzyme immunoassay procedures, where water-soluble IgG is adsorbed to the plastic plates, it was necessary to investigate the binding kinetics and equilibria of toxin binding. It is imperative that the solid phase should adsorb an adequate amount hapten in a reproducible manner, and that variability at this stage will affect the ultimate precision of the assay.

Binding of PbTx-2 (step 1 of figure 2) was evaluated in three media : ethanol, a solvent in which the toxins are reasonably soluble; phosphate buffered saline, in order to promote partitioning onto the hydrophobic polystyrene surface; and carbonate buffer of pH 9.6, which is routinely used to bind IgG to plates. Following binding, complete Protein A-urease sandwich assays (steps 2-4) were carried out. We illustrated that PBS is the most suitable medium for toxin incubation. We demonstrated the linearity of the assay with respect to reaction time, illustrating the lack of end-product inhibition of the urease system (9).

The stability of the toxin-antitoxin adsorbed on the microtitre plate (step 3), when stored in a dry atmosphere at room temperature, indicated a probable long shelf-life of the reagents . Stability curves were carried out for 2 months with no loss in activity. Regardless of the blocking agent (shown as a hatched line in steps 2,3, and 4) we used, however, substantial amounts of non-specific binding of Protein A- urease was observed. Thus, backgrounds were many times very high, contributing to a low degree of specific sensitivity. In addition, the enzyme urease, while exhibiting a high turnover number and relative insensitivity to temperature during incubation, was very sensitive to heavy metals and pH (as well as the indicator dye, which is pH sensitive). Modifications to the ELISA technique were explored last contract year, investigating (i) toxin-enzyme conjugates, (ii) commercial enzyme-anti IgG conjugates, various blocking agents for the microtitre plates, a different enzyme and substrate for visualization, and monoclonal antibrevetoxin antibodies.

We therefore sought to develop reagents utilizing a different enzyme as covalent probe, and to seek a variety of potential ELISA formats. It was described in detail in last year's annual report, and is summarized below in tabular form.

RIA. The radioimmunassay developed previously has been used almost entirely to give baseline values for ELISA protocols, which are the major thrust this year. Two potentially important developments took place last year, both involving a purification of anti-brevetoxin specific antibodies for use both in RIA and ELISA. No further specific work has been undertaken with RIA, except that the KLH-PbTx-3 immunogen has been successful in eliciting antibody production in a goat. Titers were being evaluated weekly, with a biweekly immunization schedule. Antibody titers have exceeded BSA-PbTx-3 elicited titers, based on qualitative assessment and Ouchtelony plates employing whole antigen conjugats and specific sera.

Protein G- Affinity Columns. Goat serum was purified by loading immunoglobulin solution onto a column constructed to contain 4 mg of PbTx-3 specifically bound. While specific brevetoxin binding was not increased (by comparison of protein recovered to toxin bound), the degree of non-specific color development which we observed when we used antibody purified in this manner was greatly reduced.

Brevetoxin Affinity Columns. The brevetoxin affinity column has been utilized for IgG purification following Protein G-Sepharose separation. We feel that the column is a useful tool for purification of IgG because of the specific nature of the interaction, and because the protein which did not bind possessed no specific binding affinity for radiolabeled brevetoxin. Details are given under the Results section.

Microtiter Plate Assays. Microtiter plate assays have been developed in five different ways, four utilizing antibodies and one utilizing synaptosomes. Each assay has distinct advantages and disdvantages, as described last year.

For most ELISAs, hydrophobicity can be exploited to "stick" antigen or antibody to the plate solid support. For brevetoxin microtiter plate assays, however, it was necessary to minimize nonspecific binding of toxin. Different methodologies for minimizing non-specific adsorption to the plastic plates was a specific and seemingly endless task. Table VI, which appeared last year, summarizes those findings.

Table VI. Summary of Brevetoxin Microtiter Plate Assays

Adsorbant			Sensitivity
Primary	Secondary	Tertiary	(ng/well)
FbTx-2	IgG α PbTx-3	Protein A-urease	1.0
IgG α PbTx-3	PbTx-3-urease	--------	0.001
KLH-PbTx-3	IgG α PbTx-3	r α g IgG-peroxidase	0.2
Synaptosome	PbTx-3-urease	--------	0.2
IgG α PbTx-3	PbTx-3-peroxidase	--------	0.001
Synaptosome	PbTx-3	IgG-peroxidase α PbTx-3	-----

III. Technical Approach

A. Synaptosome Binding Assay

Biological Preparation. Synaptosomes were prepared in multiple runs, generally working up material from 4-6 brains per run. Frozen brains were purchased in multiples of 30-50, and were stored at -80°C until use. Synaptosomes prepared according to the method of Dodd *et al. (10)* were stored as a pooled fraction until sufficient synaptosomes (generally from 20 brains) were prepared for multiple uses. Protein was measured on resuspended intact synaptosomes or lysed synaptosomes just prior to binding experiments using the technique of Bradford (11). Synaptosomes were stable and their results reproducible for periods of 2-3 months.

Toxins. Natural toxins were utilized as obtained. Brevetoxins were purified from laboratory cultures of *Ptychodiscus brevis*, okadaic acid was obtained from Dr. Robert Dickey at the FDA Dauphin Island laboratory, and ciguatoxic fish flesh was supplied by Dr. Thomas Tosteson at the University of Puerto Rico. Synthetic tritiated PbTx-3 was produced from PbTx-2 by chemical reduction employing cerium chloride and sodium borotritiide. Crude PbTx-3 was purified using reverse phase high performance liquid chromatography. HPLC-purified toxin had demonstrated specific acitivities of 10-15 Ci/mmole.

Binding Assays. Binding of tritiated toxin was measured using the rapid centrifugation technique (5). Binding assays were performed in a binding medium consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magneisum chloride, 5.4 mM potassium chloride, 1 mg/mL BSA, and 0.01% Emulphor EL-620 as an emulsifier for toxin.

Synaptosomes (40-80 μg total protein), suspended in 0.1 mL binding medium minus BSA were added to a reaction mixture containing tritiated PbTx-3 and other effectors in 0.9 mL binding medium in 1.5 mL polypropylene microcentrifuge tubes. After mixing and incubating at 4°C for 1 hour, samples were centrifuged (15K x g) for 2 minutes. Supernatnat solution was aspirated from each tube and the pellets were rapidly washed with several drops of a wash medium (9). Pellets were then transferred to liquid scintillation vials and bound radioactivity was measured. Non-specific binding was measured in the presence of 10 μM PbTx-3 and was substracted from total binding to yield a calculated measure of specific binding. Free tritiated probe was measured by counting an aliquot of supernatant solution prior to aspiration.

Binding Assays utilizing Solubilized Synaptosomes. The brevetoxin binding component was solubilized from pooled synaptosomes equivalent to two rats. To 2.5 mL of pooled synaptosomes, 0.3 mL aliquots of concentrated Triton X-100 buffer were added to yield a final total volume of 5 mL and containing final component concentrations as follows in pH 7.4 Tris buffer: 0.1 % Triton X-100, 0.02% Phosphatidyl choline, 50 mM choline chloride, 10 mM HEPES, 25 mM $CaCl_2$, 0.2 M NaCl, and protease inhibitors PMSF, iodoacetamide, and pepstatin A.

Solubilized binding component was centrifuged and 105,000 x g for one hour in a Ti 50 ultracentrifuge rotor, and the supernatant solution was decanted. Supernatant solutions were assayed for protein. A portion of the supernatant solution was loaded on a 1.5 a 45 cm Sephacryl S-300 size fractionation column, and the column was eluted with solubilizing buffer. Fractions were assayed for protein, and the fractions

corresponding to the molecular radius of α-subunit from sodium channel were pooled.

Solubilized samples were evaluated for purity by non-denaturing and sodium dodecyl sulfate denaturing 5-15% gradient polyacrylamide gel electrophoresis. Following electrophoresis, gels were either stained with Coomassie brilliant blue protein stain, or were transferred to nitrocellulose paper using a Western blot electrophoretic technique. Transferred protein was then silver stained. Gel electrophoresis was accompanied by molecular radius standards from 200,000 to 16,000 molecular weight.

Standard radioimmunoassays-type procedures were utilized to evaluate the specific binding of solubilized protein fractions, for subsequent use as obtained or in modified microtiter plate assays.

B. Immunoassays

Antigen. Both radioimmunoassays and enzyme-linked immunoassays utilize specific antibody against brevetoxin PbTx-3. Complete antigen construction has been previously investigated using either bovine serum albumin-covalently linked to brevetoxin, or more recently we have used keyhole limpet hemocyanin-linked brevetoxin. For this year's annual report, all of our work has utilized brevetoxin covalently linked to KLH as complete antigen. This overcomes many of our initial problems with cross-reactivity due to small amounts of BSA present in many of the assays, and KLH also elicits better antibody responses in animals.

Purified PbTx-3 was dissolved in minimal redistilled pyridine, and was succinylated with 10-fold molar excess succinic anhydride as previously described. Following separation of unreacted PbTx-3 and succinic anhydride from toxin-succinate using TLC (70/30 ethyl acetate/light petroleum), the free carboxyl function on the conjugate was covalently coupled to the ε-amino function of lysyl residues on the KLH using standard procedures (11). Following coupling, the mixture was dialyzed against PBS, pH 7.4 for overnight, and the protein concentration adjusted to yield "toxin equivalents" of 1 mg/mL.

Immunization and Antibody Purification. We continue to immunize a single goat with the KLH conjugate on alterate weeks, with bleeds on the interval weeks for assessment of toxin antibody titers. Bleeds are allowed to clot, and the serum separated by centrifugation. Antisera are treated with 0.5 volumes of saturated ammonium sulfate under conditions of stirring, and are allowed to precipitate overnight at 4°C. The precipitate is centrifuged at 3000g and the supernatant solution decanted and saved. The solution is then brought to 50% saturation by additiona of an additional 0.5 volumes of satuated ammonium sulfate, and the precipitate is allowed to form overnight, once again.

The precipitate from this centrifugation is redissolved in 0.3 volumes of original serum volume, and is dialyzed against PBS containing 0.01% sodium azide. For long term storage, the antibody solution is dialyzed against distilled water, and aliquots of approximately 25 mL volume are lyophilized. They are reconstituted in PBS pH 7.4 containing azide as needed (12).

RIA. The radioimmunoassay is as described previously, according to the method of Bigazzi *et al.* (13) to estimate serum digoxin levels. This assay is now used only to obtain correlative data for ELISA protocols.

Derivatized Toxins. Brevetoxin PbTx-3 was linked to horse radish

peroxidase, and the procedure was optimized, both with respect to reaction conditions and to stoichiometry. Derivatized materials were evaluated for stability. Attempts to link PbTx-3 to chloroperoxidase were also evaluated. PbTx-3 was also linked to urease and toxin-enzyme conjugates were evaluated.

ELISA Assays. Enzyme-linked immunosorbant assays were carried out using the derivatized toxins mentioned above, and secondary rabbit anti-goat antibody-enzyme conjugates were also evaluated for use. Western blotting of SDS gels of antibrevetoxin antibody, performed at various stages of antibody purification, were used estimate the degree of purification for each step. Only KLH antibody was utilized. Individual protocols are listed below.

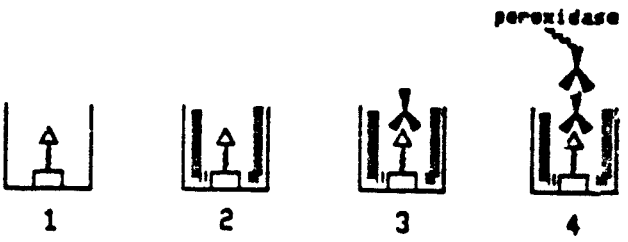

Figure 3. Specificity of rabbit anti-goat-peroxidase for goat antibody.

(1) Incubate overnight with KLH-toxin complete antigen in microtiter plates to act as primary adsorbant (Step 1). Aspirate wells and rinse 3 times with Blotto (non-fat dry milk in pH 9.6 carbonate buffer) and then block with Blotto solution for one hour (Step 2). Aspirate and add serial dilutions (X-direction) of goat antibrevetoxin (25 µg/well to 25 fg/well) (Step 3), and incubate 1 hour with goat antibrevetoxin. Aspirate and add serial dilutions (Y-direction) of commercial rabbit antigoat IgG-specific antibody (1 to 1:1000 dilution)(Step 4) and incubate 1 hour. Rinse three times with Blotto, followed by three rinses with distilled water. Add ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) reagent and peroxide, and monitor color development at 405 nm.

Microtiter plates from Costar and Dynatech were evaluated, with Immulon 1,2 and 4 being evaluated. Costar plates are uncoated plates. Immulon 1 plates are low non-specific binding plates similar to Costar plates, Immulon 2 plates are chemically treated to enhance protein uptake with low variance, and Immulon 4 plates are treated to give the "maximum" protein uptake with minimal variance.

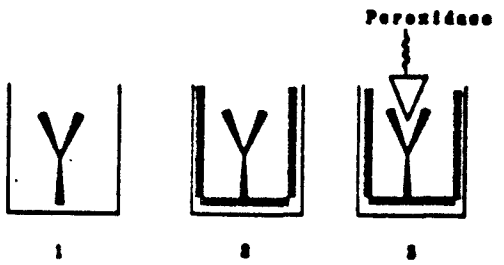

Figure 4. Assays using toxin-peroxidase covalent conjugates.

(2) This type of assay was anticipated to be of use in "column"-type formats, where toxin-peroxidase had been presorbed to antibody immobilized on a solid-support. Subsequent exposure to free toxin would in theory release toxin-peroxidase conjugate from the antibody.

For synthesis of the toxin-peroxidase conjugate, several different stoichiometries were employed for the coupling reaction. Conjugate protocols utilized in the carbodiimide coupling reaction were repeated to produce toxin-protein conjugates similar to those used for immunization. Following coupling using tracer tritiated PbTx-3, conjugates were evaluated on SDS polyacrylamide gels. Gels were stained with Coomassie blue protein reagent, and bands were cut from the gel and the radioactivity assessed. Gels were also Western blotted, and the blotted proteins were probed with goat antitoxin followed by rabbit antigoat alkaline phosphatase. Toxin-HRP enzyme activity was measured and compared to unconjugated enzyme. Stability of the conjugate was evaluated under various regimes.

Checkerboard antibody versus toxin-peroxidase concentrations were carried out in microtiter plates. Once again, buffer character and time of incubation at each step were evaluated. Competition assays were carried out using unlabeled PbTx-3 as competitor, using optimized antibody and toxin-peroxidase concentrations.

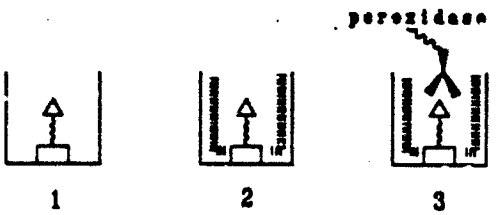

Figure 5. Assays using antibrevetoxin covalently linked to peroxidase.

(3) Goat polyclonal antibodies were purified using ammonium sulfate precipitation, followed by dialysis, protein G-chromatography, and finally brevetoxin affinity column chromatography. In this manner, we begin with purified reagents for construction of the antibody-peroxidase conjugate. Goat anti-brevetoxin antibodies were coupled to horse radish peroxidase using the standard sodium meta-periodate method (14).

Checkerboard assays employing dilutions of KLH-toxin conjugates and dilutions of antibrevetoxin-peroxidase were attempted using a variety of times for incubation, and several potential blocking solutions. Microtiter plates were selected to display minimal specific protein binding (because the toxins were used as primary adsorbant), thereby minimizing non-specific IgG binding to the plates.

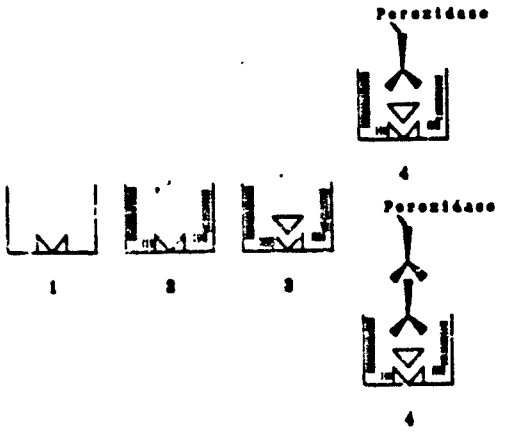

Figure 6. Assays employing excitable membrane components.

(4) The brevetoxin binding component in rat brain synaptosomes was evaluated as a potential microtiter plate reagent, first attempting to utilize synaptosomes themselves, adsorbed to platic plates, and second by identifying the brevetoxin binding component in synaptosomes biochemically--isolating it and purifying prior to use. In all cases, the two protocols depicted in Figure 6 were explored. The assays depend on the initial binding of a brevetoxin-specific binding component as indicated in step #1. This is followed by a standard blocking step to minimize additional protein binding unless it is specific to the reaction sequence (step #2). Step #3 involves the addition of brevetoxin in standard dilutions, followed by the additional of either anti-brevetoxin peroxidase (top panel of step #4) or sandwich assays involving sequential addition of goat antibrevetoxin followed by rabbit antigoat-peroxidase conjugate. The latter rabbit antigoat peroxidase allowed us to evaluate specific antibrevetoxin binding in initial protocols.

IV. Results and Discussion

A. Synaptosomes

Stability. For experimental work to proceed utilizing an homogeneous and reproducible preparation, we developed a procedure which allows for the preparation of 20-50 rat brains, storage of synaptosomes at -80°C, and use over a period of days to weeks of small aliquots of synaptosome preparation. The availability of whole unstripped rat brain from Harlan Sprague Dawley Inc in Indiana allowed us to work-up approximately 6-8 times the number of rat brains at the same over-all cost. Brains are worked up according to the method of Dodd *et al.* (10), and are stored as synaptosomes in serum binding medium SBM (5) in 5 mL aliquots. Procedurally, for each brain prepared (10 mL total final volume), 0.5 mL of the preparation is aliquoted into 20 tubes and frozen. Each group of brains is prepared in this manner, adding 0.5 mL equivalents per brain to each of the twenty tubes. When tube volumes reach the 5 mL volume, the aliquots are stored as a batch of twenty tubes, each of 5 mL volume and equivalent in sample. Each tube, when thawed for use, will allow for the preparation of a "two-tray" experiment, i.e. one standard displacement or competition study. Using this procedure, we are able to reproduce results within a batch with very low standard errors and

deviation, and batch to batch variation is likewise very low. We could detect no change in either dissociation constant or binding maximum using synaptosomes prepared in this manner, when compared with freshly prepared material.

Solubilization of the Brevetoxin Binding Site. Seven different protocols and one control protocol were utilized to investigate the binding of tritiated brevetoxin PbTx-3 to solubilized synaptosomes, each utilizing the modified protocol of Bigazzi *et al.* (13). These are listed in Table VII.

Table VII.
Binding Assay Method Alternatives
Using Solubilized Synaptosomes

Protocol #	Specific Protocol	DPM	% Of Total Binding
#1	a->d->g->e->h	82K	100.0
#2	a+b->d->g->e->h	33K	40.2
#3	a+c->d->g->e->h	29K	35.4
#4	a->d->g->e->f->g->e->h	15K	18.3
#5	a->d->g->e->f->g->e->f->g->e->h	26K	31.7
#6	a->d->g->e->f->b->g->e->h	35K	42.7
#7	a->d->g->e->f->c->g->e->h	31K	37.8
#8	a->d->2 x volume g->e->h	12K	14.6

a=65 μg synaptosomal protein + 10 nM tritiated PbTx-3 in SBM; b=10 μM unlabeled PbTx-3; c=1.0 μM unlabeled PbTx-3; d=incubation for 60 minutes at 4°C; e=centrifugation at 15,000 x g; f=transfer supernatant solution to a fresh tube; g=1% charcoal suspension in SBM; h=liquid scintillation counting of an aliquot of supernatant solution.

Resuspension 2 times (#8) appeared to reduce specific binding. One wash (#4) only gave about 50% of the specific binding present in the control tube (#1). Two washes (#5) yielded approximately the same amount of specific binding as did the control containing 10 μM unlabeled toxin (#2). Three washes (#6) produced slightly higher values than control values, but may result from washout of non-specific binding. One wash, followed by 10 μM unlabeled toxin appeared to yield very good values (#7), 31K versus 33K in controls. Solubilized synaptosomes incubated with 1 μM unlabeled toxin (#3) gave very close values to the specific binding measured in tube #2. Protocols #2 and #7 were optimized for further use in soluble receptor assays for brevetoxin.

B. Antibodies

Stability. Following ammonium sulfate precipitation, dialysis against distilled water, and lyophilization, anti-brevetoxin IgG fraction is stable indefinitely at -20°C in sealed serum bottles. We have been able to detect no difference in specific tritiated brevetoxin binding with age of stored material. Lyophilized material theoretically should be stable at room temperature so prepared, but because of the limited amounts of specific antibody available the study does not presently warrant

investigation. Preparation of antibody to this step allows for an approximately 15-fold purification, and a reduction in storage volume from 4 liters to 500 mL in ten separate bottles.

Affinity Purification of Antibody. From crude serum, which exhibited about 15 units specific brevetoxin binding equivalents per milligram protein, and ammonium sulfate precipitation which increased specific binding to 158.8 units per milligram protein, we subjected antibrevetoxin antibody to sequential protein G- and brevetoxin-specific affinity chromatography. Overall, 29.5% of the specific binding equivlanets were recovered through purification, with a final specific binding of 307 units per milligram protein (Table VIII).

Table VIII.
Antibrevetoxin Antibody Purification
from
Goat Serum

Preparation	Protein (mg)	Units	% Units	Specific Binding (U/mg Protein)
Serum	500	7500	100	15
Ammonium Sulfate	43	6828	91	158.8
Protein G Affinity	20.8	5254	70	252.6
Brevetoxin Affinity	7.2	2210	29.5	307

At this time, all ammonium sulfate precipitate is being prepared through the protein G affinity column, and shall be pooled as a purified IgG fraction. We hesitate to move on through the brevetoxin affinity column for this step has not been optimized and several problems still exist. As an example, we lose approximately 40% of the specifically bound antibrevetoxin IgG through this step. The lost IgG cannot be recovered by increasing salt concentrations or detergent through the column (i.e. it is not bound to the column) and we do not lose binding affinity by irreversibly binding to the toxin on the column (columns do not appear to lose specific binding capacity from multiple runs).

It is also unlikely that the specific brevetoxin binding component is not IgG, for the loss occurs on the toxin affinity column, following elution from the Protein G column. Finally, the total protein recovery from the toxin affinity column is 24 mg from 24.4 mg loaded. The protein which does not adsorb to the column (16.8 mg) also does not contain any specific brevetoxin binding demonstrable. Specific antibrevetoxin antibody purified from toxin affinity columns was dialzyed against pH 7.4 phosphate buffered saline and 0.02% sodium azide for 24 hours, followed by dialysis against distilled water. Fractions were lyophilized for further use in ELISA development.

Regardless of the short-comings in specific IgG purification, and loss of some toxin binding activity, material which is specifically adsorbed and desorbed from brevetoxin affinity columns is substantially better for development of microtiter plate assays, as will be demonstrated later in this report. Th specific antibody is also an excellent reagent for locating brevetoxin photoaffinity probes on voltage-sensitive sodium

channels, following SDS gel electrophoresis and Western blotting (15). Specific binding affinity ranges from 80-90 % in purified preparations. The dissociation constant for brevetoxin-specific antibody is 1.32 nM, with an exhibited binding maximum of 17.7 pmoles/mg protein in crude serum (2.8 μmole toxin/mmole antibody) or about 58 μmoles toxin/mmole antibody in brevetoxin affinity column purified material.

In general, the variability in affinity constants for antibody-antigen interaction is extremely variable, and literature reports range from 10^5 mol^{-1} to 10^{12} mol^{-1}. However, because polyclonal antibody preparations are by their nature derived from multiple clones, each producing their own unique antibody molecule, the measured affinity constant is a composite of all affinity constants in the mixture. Thus, the composite dissociation constant for our antibrevetoxin antibodies is in mid-range of the values reported in the literature, in the nanomolar range.

It is fairly obvious from our immunization results, that the brevetoxins are not particularly good antigens themselves, although their antigenicity can be improved substantially by covalent coupling to KLH, or to a lesser degree BSA. However, even under immunization conditions using these covalent conjugates, antibody response is relatively poor. One well known reason that animals fail to elicit strong antibody responses pertains to recognition of self, i.e. that the appropriate B and/or T-cell clones have been eliminated during self-tolerance recognition. In other words, if the toxins represent a class of compounds which are recognized as "self", they will not elicit antibody production. Although we feel this possibiity is highly unlikely, we have no experimental evidence to rule it out.

A second more likely reason for immunological tolerance to the brevetoxin conjugates centers around the potential failure of class II proteins (on cell surfaces) to bind to the antigen fragments. Since there are only about 40 class II proteins which are manufactured by any one animal, individuals may not possess the proper class II protein. We felt we could overcome failure of class II recognition by coupling to KLH, and indeed titers were better using this carrier protein. However, if toxin fragments are required for continuation of the proper clones, we are faced with a continual problem.

The low titers we achieved were partially remedied by purification of the specific antibrevetoxin antibodies and concentration of that fraction of serum. The avidity of the complex, i.e. the overall stability of the antibody-antigen interaction, is rather low, which is exploitable in terms of competition assays for toxin detection. Radioactive toxin-antibody complexes can be easily perturbed by unlabeled toxin, whether the unlabeled material is added at the same time as the radioisotope, or later after complex formation. This allows for the development of true "displacement" assays, as opposed to the more conventional "competition assays".

Cross-reactivity with Other Polyether Toxins in RIA. In our previous Annual Report, we described the interaction of antibrevetoxin antibodies with the suite of naturally-occurring and synthetic brevetoxins at our disposal. We described the higher sensitivity of detection of the Type-1 backbone versus Type-2 backbone, and postulated a bit about the potential for cross-reactivity with other polyether dinoflagellate toxins like ciguatoxin and okadaic acid. However, those materials were unavailable to us, and we had no prospect of obtaining them.

Okadaic acid was supplied by Dr. Robert Dickey (FDA Dauphin Island Laboratory) in purified form, and the progenitor organism was supplied by Dr. Carmelo Tomas (Florida Department of Natural Resources Marine Research Institute). Either dinoflagellate extract, dinoflagellate cell, or purified okadaic acid were utilized in assays (figure 7). Crude ciguatoxin, isolated from ciguatoxic barracuda was supplied by Dr. Thomas Tosteson of the University of Puerto Rico. Radioimmunoassay was done on this material following high performance liquid chromatography; 10 minute fractions were collected for 60 minutes and were correlated with mouse bioassay (figure 8).

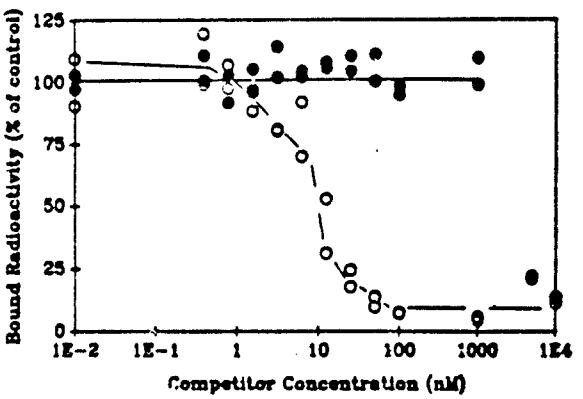

Figure 7. Displacement Brevetoxin Radioimmunoassay for Okadaic Acid (closed circles), and brevetoxin control (open circle).

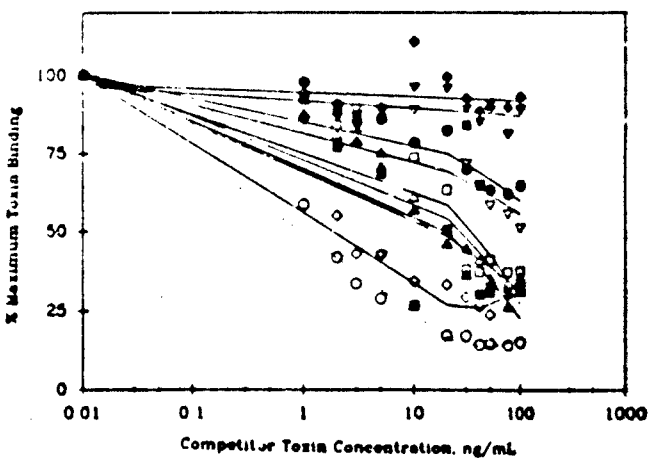

Figure 8. Brevetoxin Radioimmunoassay Using HPLC Fractions from Ciguatoxic Barracuda. Brevetoxin PbTx-3 (open circles), orally lethal fraction #2 (10-20 minute elution, open diamond), fractions #3,4,and 5 (22-30, 30-40, 40-50 minutes; open invert triangle, closed squares, and closed triangle respectively), orally intoxicating but not lethal, and control incubations with okadaic acid (closed circles), methanol vehicle (closed invert triangle), and fraction #6 (50-60 minutes, closed diamond).

C. Derivatives

Toxin Enzyme Conjugates. Brevetoxins linked to either Jack Bean urease or Horse Radish peroxidase were evaluated as specific probes in immunoassays. These toxin-enzyme conjugates were prepared as succinate linked materials as described in materials and methods. For urease conjugates, 1:2, 1:4 and 1:6 molar ratios were investigated. The following protocol yielded the most reproducible results:

[1] prepare succinylated PbTx-3 as previously described (9);

[2] dissolve succinylated PbTx-3 in minimal 50% pyridine and add 2 mg EDC carbodiimide for every mg succinylated toxin (about 10-fold excess EDC);

[3] after 2 hr mixing at room temperature, add succinate toxin to a 5.7-fold excess urease in 1 volume equivalent of water, in three equal aliquots. Total volume after addition is 2-fold the volume in step [2], final pyridine concentration 25%;

[4] stir at room temperature overnight;

[5] transfer to dialysis tubing (MW exclusion 10,000) and dialyze against 3 changes of pH 7.4 phosphate buffered saline.

For peroxidase conjugates, toxin:enzyme molar ratios of 1:1, 1:2, 1:4, and 1:6 were evaluated. The following protocol yielded the most reproducible results:

[1] prepare succinylated toxin as previously described (9);

[2] dissolve succinylated PbTx-3 in minimal 50% pyridine and add a 2:1 mg ratio of EDC carbodiimide relative to toxin (again a 10-fold excess);

[3] stir at room temperature overnight;

[4] after mixing overnight, mix enough horse radish peroxidase in water to so that when the toxin succinate is added, the final ratio of 1:6 molar toxin:HRP coupling mixture (0.25 mg toxin succinate to 90 mg HRP) will be achieved;

[5] stir at room temperature 2 hours and at 4°C overnight;

[6] dialyze as in urease assay.

In all cases it is imperative to add toxin-succinate- carbodiimide to enzyme and not enzyme to hapten. In this manner, maximum enzyme activity is maintained by limiting the number of haptens linked per enzyme.

Stability of Derivatized Toxin-Enzyme Conjugates. As outlined in detail last year, toxin-urease conjugates lose activity with time, even in the refrigerator or at freezer temperatures. Several sequential batches of toxin-urease (1:5.7) became inactive (no enzyme activity but still protein and toxin present at proper stoichiometry) very quickly and within a couple of weeks exhibited less than 10% of the assayed activity at the time of synthesis and dialysis. We are uncertain as to the cause of the inactivation. Based on our previous experience with uncoupled urease and its sensitivity to heavy metals and temperature regimes, we initially began to develop and utilize reagent buffers which contained low concentrations of heavy metals (i.e. reagent or ACS grade or better). However, we began to doubt the utility of an urease assay, especially when

considering the various and multiple contaminants which might be present in actual samples. Aside from figure 7 reported in our last year's Annual Summary Report (4), we shall report no further on urease-toxin assays. Thus, with this enzyme system we were unsuccessful in our attempt, even though we could demonstrate a dose-dependent displacement of toxin-enzyme conjugate while conjugates remained active (14).

Assays employing HRP were much more readily adaptable and useable owing to the inherent stability of peroxidase conjugates. Our initial results using toxin-peroxidase in 50% glycerol were not encouraging, and much of the enzyme activity was lost in a period of days, as in the urease case without loss of either protein or toxin. Thus, something was happening to the enzymatic activity. Six different storage protocols for toxin-enzyme conjugate were tested: [1] speed vacuum-dried (Savant) preparation with 0.1% BSA, frozen and dessicated; [2] speed vacuum-dried with no BSA, frozen and dessicated; [3] speed vacuum-dried with 0.5% BSA, room temperature dessicated; [4] speed vacuum dried with no BSA at room temperature dessicated; [5] frozen in solution with 0.1% BSA; and [6] frozen with 0.1% BSA plus 50% glycerol.

Based on recovery of enzymatic activity, protocol [3] was best for maintaining integrity of the preparation, followed by [5] and [6]. These recoveries indicate that during coupling of toxin to peroxidase, approximately 40% of the peroxidase activity is retained.

Following storage under conditions of [3], 15% SDS polyacrylamide gel electrophoresis was performed on the derivatized toxin-enzyme, and the migration was compared with that of unconjugated toxin or enzyme. Coomassie brilliant blue staining of developed gels indicated unreacted HRP to possess a molecular radius of about 44,000 Daltons. Unconjugated toxin did not visualize with staining--as was expected. Toxin-peroxidase conjugates possess a molecular radius of about 48,000 to 54,000 Daltons, with no 44,000 molecular weight material remaining. Thus, we are certain we have conjugated the toxin to the enzyme, and believe that the 40% activity retained is due to a 60% reduction in enzymatic activity caused by toxin conjugation---and not due to 100% reduction in 60% of the enzyme due to conjugation, with the remaining 40% activity arising from that portion of the enzyme preparation not conjugated. This point is <u>critical</u> to assay development, and has been demonstrated.

Western blotting of the SDS gel, and subsequent ABTS substrate incubation, indicates that the peroxidase with altered mobility on gels is indeed still active. This fact, when compared with gels cut up and assayed for tritium counts associated with added tracer PbTx-3 toxin, and silver stained Western blots, indicates that enzyme activity, toxin, and protein all comigrate in SDS gels and are distinct from unlatered enzyme or toxin.

<u>Stability of Antibrevetoxin-Antibody Linked to Peroxidase.</u> This substrate is a conventional one, in deference to those already outlined. The antitoxin-peroxidase conjugate was determined to be very stable, on the order of the stability of the enzyme itself. Strong signals in ELISA's (next section) indicated about 1:1 conjugation ratio for antibody to enzyme and allowed for a 1:20,000 diltuion of this reagent. Yields for this periodate conjugation reaction were low however, adjudged to be about 35%, and usefullness of the reagent was directly correlated with the purity of the antibody utilized for coupling. Only ammonium sulfate precipitated antibody had been used for coupling, and at that time high quality HRP-linked rabbit antigoat antibody became available. Thus

although the feasibility is evident for antibrevetoxin-peroxidase probes, the use of antispecies-peroxidase conjugates allows for reproducibility and convenience, especially when coupled with the amplification possible with sandwich assays. Antispecies antibodies linked to enzymes have been extremely stable in our hands.

D. Enzyme-Linked Immunoassays

No data will be given for protein A ELISA, owing to the low degree of affinity exhibited by Protein A-urease for the antibrevetoxin antibody. For each of the enzyme-immunoassay formats, a single figure will be presented with the protocol summarized in the legend.

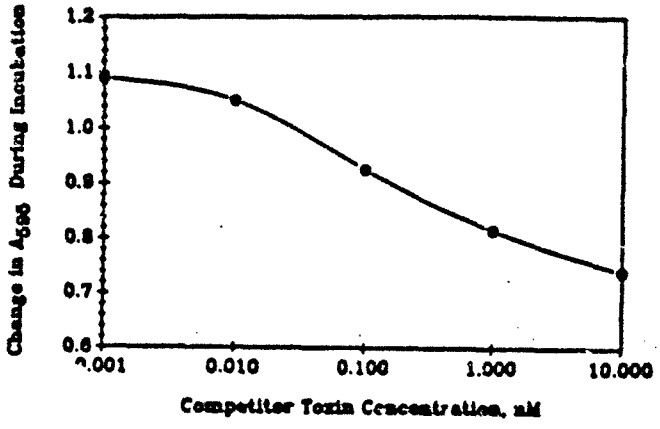

Figure 9. Toxin-Urease Assay. This assay is a modification of the toxin-peroxidase assay presented earlier in figure 4. Assays were conducted at room temperature, measuring the change in absorbance at 595 nm using a microtiter plate reader. Primary adsorbant was 4 ng antibrevetoxin IgG per well; toxin-urease as secondary reagent at 8 μg urease protein per well; Brevetoxin PbTx-3 as competitive toxin at concentrations ranging from 0.001 to 10.0 nM.

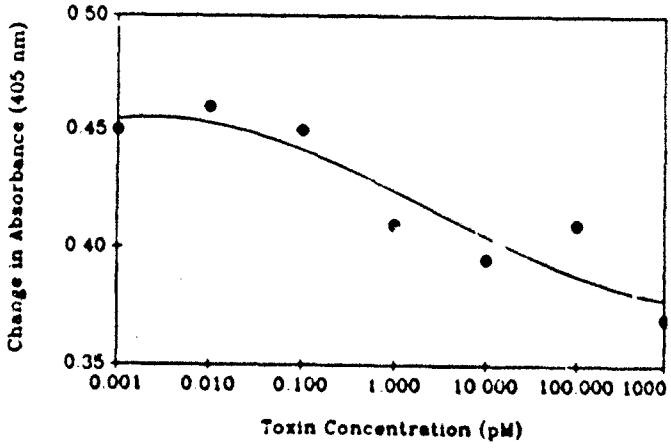

Figure 10. Toxin-Peroxidase Assay. Assays were conducted at room temperature, measuring ABTS conversion at 405 nm. Primary adsorbant was 70 μg goat antibody per well; secondary reagent was 35 μg toxin-peroxidase per well; PbTx-3 competitor concentrations range 0.001 pM to 1 nM.

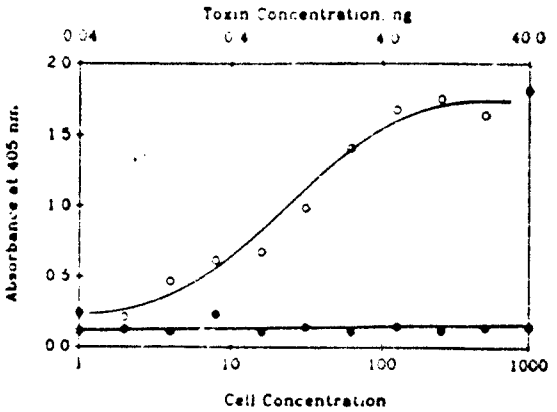

Figure 11. Non-Competitive Peroxidase-Linked Sandwich Immunoassay. Assays were conducted at room temperature, measuring ABTS substrate conversion at 405 nm according to the protocol listed illustrated in figure 3. Samples of *P. brevis* cells, *Prorocentrum lima* cells, okadaic acid, or brevetoxin PbTx-3 were incubated 1 hour as primary adsorbants. Following a 1 hr Blotto blocking of non-specific binding sites, 30 µg antibrevetoxin protein G purified IgG was added to each well for a 1 hr incubation. Rabbit anti-goat IgG linked to peroxidase was added as tertiary adsorbant (1:1000 to 1:5000 diltuion of commercial preparation). After addition of ABTS substrate, changes in absorbance were measured for a two hour period, over which period color development was linear. A derived value per hour was calculated. Cell concentrations for dinoflagellates are quantified on the lower X-axis, and toxin concentrations are quantified on the upper X-axis. Brevetoxins and *P. brevis* cell extracts are illutrated by the open circles. Okadaic acid and *P. lima* cell extracts are illustrated in closed circles.

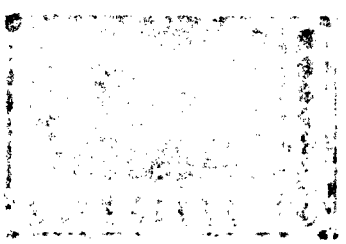

Figure 12. Checkerboard Protocol of Peroxidase-Linked Sandwich ELISA. Increasing dilutions of rabbit anti-goat-peroxidase were prepared in the Y-direction, as tertiary adsorbant to optimize detection of brevetoxins. Decreasing concentrations of PbTx-3 secondary adsorbant at a fixed 1:80 dilution of primary goat antibrevetoxin antibody were prepared in the X-direction. At a working dilution of 1:1000 (fourth row down from top), the color development curve shown in open circles in figure 11 was generated.

Thus, most of the assays explored could detect brevetoxins in a quantitative manner. There was a substantial difference in their sensitivity to differences in brevetoxin concentration, however. In cases of "displacement assays", i.e. assays which employed toxin conjugated to enzyme which was first adsorbed to specific antibody, a low response factor was always evident. In the case of toxin-urease assays, there was a differential absorbance change of 0.4 AUFS over four orders of magnitude toxin concentration. This was an unacceptable colormetric assay, and could not be improved upon. The non-specific color development was unduely high as well.

Toxin-peroxidase assays were of likewise low differential color development, there being a change in color development of 0.1 over six orders of magnitude toxin concentration. However, assays employing peroxidase were pursued owing to the very low degree of color development using ABTS substrate. Peroxidase assays employing guaiacol or ortho-dianisidine had characteristically high background color development and were not pursued.

Assays employing affinity purified antibrevetoxin antibody were far superior to any of those employing ammonium sulfate precipitated or even Protein G purified antibody. Using rabbit antigoat-peroxidase commerical conjugate preparation, we achieved the highest degree of success to date. The assay has sufficient promise to pursue, for several reasonr: [1] the rabbit antigoat-peroxidase conjugate and the ABTS substrate are reproducible preparatins which can be conveniently purchased from several sources; [2] goat antibody is readily detected using this conjugate and several anti-species antibodies can bind, forming multimeric complexes which increases sensitivity; [3] the assay is a direct assay in deference to a competitive assay, i.e. more toxin in the sample leads to more color development; [4] the assay is linearly quantifiable from 20 pg to 4 ng toxin concentration in 0.1 ml volume. This is roughly the orders of magnitude which would be present during intoxication phenomena; [5] each of the reagents can be stored under separate conditions and assay kits do not require assembly prior to running assays; and [6] the initial step in the assay is adsorption of toxin to hydrophobic plastic plates. The non-polar nature of this interaction maximizes toxin adsorption, and non-coated low protein binding plates (the cheapest ones available) suffice for assays.

Thus, sandwich assays of this type are being optimized to complete the work scope under this contract, and we expect, by the time the Final Report is complete for C-7001, that we will have further information on the items listed under Recommendations.

V. Conclusions

[1] Antibodies to brevetoxin-protein conjugates can be raised in goats;

[2] Keyhole limpet hemocyanin conjugates are superior to bovine serum albumin conjugates;

[3] Although titers are not high in serum, antibody concentration can be effectively increased by ammonium sulfate precipitation, followed by Protein G affinity chromatography;

[4] Brevetoxin specific antibody can be isolated by using brevetoxin covalent affinity columns as purification matrix;

[5] Radioimmunoassays can be used to evaluate serum titers of antibody, and to detect unknown concentrations of toxin by competitive immunoassay;

[6] RIA's do not cross-react with okadaic acid, but do cross-react with ciguatoxin isolated from toxic barracuda fish flesh;

[7] Enzyme-immunoassays can be developed for polyether toxins;

[8] Toxin-enzyme conjugates, although technically feasible, are not practical due to lowered enzyme activity and instability;

[9] Antibrevetoxin antibodies linked to peroxidase can be prepared and assays developed using this as reagent;

[10] Immunoassays using a secondary antibody linked to an enzyme are optimal for use in detecting goat antibrevetoxin antibody attached to toxin;

[11] Antigoat antibodies linked to peroxidase lead to enhanced sensitivity due to multimeric complexes (and hence multiple peroxidase enzymes) which can form. These multimeric complexes lead to greater enzyme activity at lower toxin concentrations;

[12] Final disposition of sodium channel based mictrotiter plate assays and conclusions pertaining to detection in biological fluids are being evaluated and will be summarized in the Final Report.

VI. Recommendations

[1] Complete evaluation of brevetoxin-->goat antibrevetoxin--->rabbit antigoat IgG linked to peroxidase--->ABTS color development sandwich immunoassays;

[2] Investigate further the use of sodium channel components in development of sandwich assays;

[3] Explore the quantitative reduction in background and increase in sensitivity of assays using brevetoxin-column affinity purified antibodies;

[4] Examine antibody-synaptosome competition for toxin binding.

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